

A Purple Acid Phosphatase from Sweet Potato Contains an Antiferromagnetically Coupled Binuclear Fe-Mn Center*[§]

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A purple acid phosphatase from sweet potato is the first reported example of a protein containing an enzymatically active binuclear Fe-Mn center. Multifield saturation magnetization data over a temperature range of 2 to 200 K indicates that this center is strongly antiferromagnetically coupled. Metal ion analysis shows an excess of iron over manganese. Low temperature EPR spectra reveal only resonances characteristic of high spin Fe(III) centers (Fe(III)-apo and Fe(III)-Zn(II)) and adventitious Cu(II) centers. There were no resonances from either Mn(II) or binuclear Fe-Mn centers. Together with a comparison of spectral properties and sequence homologies between known purple acid phosphatases, the enzymatic and spectroscopic data strongly indicate the presence of catalytic Fe(III)-Mn(II) centers in the active site of the sweet potato enzyme. Because of the strong antiferromagnetism it is likely that the metal ions in the sweet potato enzyme are linked via a μ -oxo bridge, in contrast to other known purple acid phosphatases in which a μ -hydroxo bridge is present. Differences in metal ion composition and bridging may affect substrate specificities leading to the biological function of different purple acid phosphatases.

Purple acid phosphatases comprise a family of binuclear metal-containing enzymes, the members of which have been identified in plants, animals, and fungi. The animal enzymes contain an antiferromagnetically coupled binuclear iron center. The active form of the enzyme (Fe(III)-Fe(II)) exhibits a distinctive EPR signal with principal *g* values of 1.93, 1.75, and 1.50 (1), whereas the inactive (Fe(III)-Fe(III)) form is EPR-silent at X-band microwave frequencies (2, 3). Plant purple acid phosphatases appear to be more diverse. Red kidney bean purple acid phosphatase, the best characterized plant enzyme, contains an Fe(III)-Zn(II) center (4). Replacement of Zn(II) by Fe(II) yields an enzyme with full activity and with spectral properties very similar to those of the animal enzymes (5, 6).

The sequences of three cDNAs encoding different isoforms of sweet potato purple acid phosphatase have recently been reported (7, 8). Two of these isoforms have been purified. One form was shown by Durmus *et al.* (7) to contain one *g*-atom of

iron and 0.77 *g*-atom of zinc/subunit. Comparison of its spectroscopic properties with those of the red kidney bean enzyme indicated that the metal ions are present as a binuclear Fe(III)-Zn(II) center (7). A different form exhibiting 66% sequence identity to the Fe(III)-Zn(II) enzyme has recently been characterized in our laboratory. It contains one *g*-atom of iron and 0.6–0.8 *g*-atom of manganese/subunit (8). We now report enzymatic, magnetic susceptibility and EPR studies on this enzyme demonstrating that the enzyme contains a catalytically active Fe(III)-Mn(II) center that is strongly antiferromagnetically coupled, providing the first reported evidence for such a center in a protein.

EXPERIMENTAL PROCEDURES

Purification and Enzyme Assay—The enzyme was purified by a combination of juice extraction, acetone and ammonium sulfate fractionation, DEAE-cellulose chromatography at pH 7.0, and gel filtration on a Sephadex G-150 Superfine column at pH 4.90 as described elsewhere (8). The enzyme was purple when concentrated and exhibited essentially the same visible absorption spectrum ($\lambda_{\text{max}} = 560$ nm, $\epsilon_{\text{max}} = 3207$ M⁻¹ cm⁻¹) as the red kidney bean enzyme ($\lambda_{\text{max}} = 560$ nm, $\epsilon_{\text{max}} = 3360$ M⁻¹ cm⁻¹) (4). Enzyme assays were performed at 25 °C using *p*-nitrophenyl phosphate (5 mM) as substrate in 0.1 M acetate buffer, pH 4.90. The specific activity of the sample used for magnetic susceptibility and EPR measurements was 675 units/mg. The protein subunit concentration was 0.556 mM (based on a subunit weight of 55 kDa and an $A^{1\%}_{1\text{cm}}$ at 280 nm of 27.03 (8)).

Metal Ion Analysis—Metal ion content was determined by inductively coupled plasma mass spectrometry using a PerkinElmer SCIEX-ELAN 5000 spectrometer. Samples and standards were prepared in 0.1% HNO₃. Separate standard curves were routinely prepared for iron, zinc, copper, and manganese. Samples were measured in quadruplicate. Metal ion analysis showed that the sample of enzyme used for magnetic susceptibility and EPR measurements contained 1.035 ± 0.108 iron, 0.582 ± 0.044 manganese, 0.183 ± 0.019 zinc, and 0.11 ± 0.028 copper/subunit of 55 kDa.

Oxidation and Reduction—Pig purple acid phosphatase was prepared as described previously (9). Protein concentrations for the oxidation and reduction experiments were 300 μ M for the pig enzyme and 300 μ M (subunit concentration) for the sweet potato enzyme. Oxidation was carried out by incubating the enzymes in 0.1 M acetate buffer, pH 4.90, containing 30 mM H₂O₂ at 4 °C for 45 min followed by removal of the peroxide by gel filtration. Reduction of oxidized pig purple acid phosphatase and untreated sweet potato enzyme was carried out by incubation in pH 4.90 buffer containing 140 mM β -mercaptoethanol at 25 °C for 150 min followed by gel filtration. The specific activities of the samples were determined using the assay described above.

Partial Removal of Metal Ions—Sweet potato purple acid phosphatase (40 μ M) in 0.1 M acetate buffer (pH 4.9) was mixed with pyridine-2,6-dicarboxylate (2 mM) at 20 °C (5). An aliquot of freshly prepared sodium dithionite solution (10 mM) was added. After a short incubation (10–60 s) the mixture was separated on a Sephadex G-25 column, which was equilibrated with 0.1 M acetate buffer (pH 4.9).

Multifield Saturation Magnetization—Multifield saturation magnetization (10, 11) data were collected with a Quantum Design MPMS SQUID magnetometer and corrected for the magnetization of the deu-

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[§] The on-line version of this article (available at <http://www.jbc.org>) contains Fig. S1.

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terated acetate buffer (pD 4.9, 0.1 M), the effects of mismatches in residual ferromagnetic impurities in the quartz sample holder (volume = 0.146 ml), background diamagnetism of the sample, and temperature-independent paramagnetism (11). Fitting of the data employed the program WMAG (11) and an $S = 5/2$ spin Hamiltonian. To reduce the number of variables, the g and E/D values were held at 2.0 and 0.1925, respectively, as deduced from the EPR spectrum. Reasonably good fits, as judged by the "goodness of fit" parameter in the program, were obtained for $|D|$ values between 0.8 and 1.2 cm^{-1} .¹

EPR Spectroscopy—X-band (9–10 GHz, TE_{102} rectangular cavity) EPR spectra were measured on Bruker EPR spectrometers (ESP300E and Elexsys E500). Low temperatures (1.5–2.5 K) were obtained with a flow-through Oxford instruments ESR910 cryostat in conjunction with an Oxford instruments ITC-4 variable temperature controller. Spectrometer tuning, signal averaging, and subsequent data manipulation were performed with either Bruker esp300e (v3.02) software for the ESP300E spectrometer or Xepr[®] (v2.0) for the Elexsys spectrometer. Instrument settings were as follows: microwave power, 20 mW; modulation frequency, 100 kHz, and the modulation amplitude was always less than one-tenth of the linewidth at half-height. The microwave frequency and magnetic field were calibrated using an EIP 548B microwave frequency counter and a Bruker ER035 M gaussmeter, respectively.

Computer simulation of the EPR spectra was performed using version 1.0 of XSophe[®] (13–15) running on a SGI R5K O2 work station. Matrix diagonalization was employed within Sophe (13) for the calculation of the eigenvalues/eigenvectors, which were used to determine the resonant field positions and transition probabilities. The field-swept EPR spectral intensity was normalized with the factor $(d\omega/dB)$. Spectral comparisons of the simulated and experimental spectra were performed with Xepr[®] running on the SGI work station.

RESULTS AND DISCUSSION

Several independent preparations of sweet potato purple acid phosphatase, including the one used in the present study (see "Experimental Procedures"), contained essentially stoichiometric amounts of iron, 0.6–0.8 g-atoms of manganese, and smaller amounts of zinc (<0.05–0.18 g-atoms) and copper (0.08–0.11 g-atoms)/subunit of molecular mass 55 kDa (8). In purple acid phosphatases from red kidney bean, soybean, and one isoform from sweet potato, where the active form of the enzyme contains an Fe(III)-Zn(II) center, the content of the divalent metal ion is also substoichiometric, ranging from 0.6 to 0.8 g-atom/subunit (4, 7, 8). In all three enzymes the iron content is stoichiometric, reflecting tighter binding of the trivalent metal ion. For the red kidney bean and soybean purple acid phosphatases, it could be shown that the specific activity correlates with the content of the divalent ion, Zn (4, 8). Furthermore, smaller amounts of manganese, which are present in preparations of the soybean enzyme, are readily removed by dialysis against 5 mM EDTA without effecting a change in specific activity. A similar treatment with EDTA of the sweet potato enzyme characterized in our laboratory does not remove manganese, suggesting that the Fe-Mn binuclear cluster in sweet potato purple acid phosphatase is responsible for the catalytic activity. To substantiate this conclusion, the manganese and zinc contents were correlated with the enzyme's specific activity.

First, prolonged storage of sweet potato purple acid phosphatase at 4 °C results in a loss of manganese with a proportional decrease in specific activity. For example, when enzyme containing 1.0 g-atom of iron and 0.63 g-atom of manganese and having a specific activity of 730 units/mg was stored for one year at 4 °C, the manganese content decreased to 0.47 g-atom/subunit and the specific activity decreased to 500 units/mg. The zinc content remained invariant. Upon incubation with Mn(II) the manganese content increased to 0.71 g-atom/subunit and

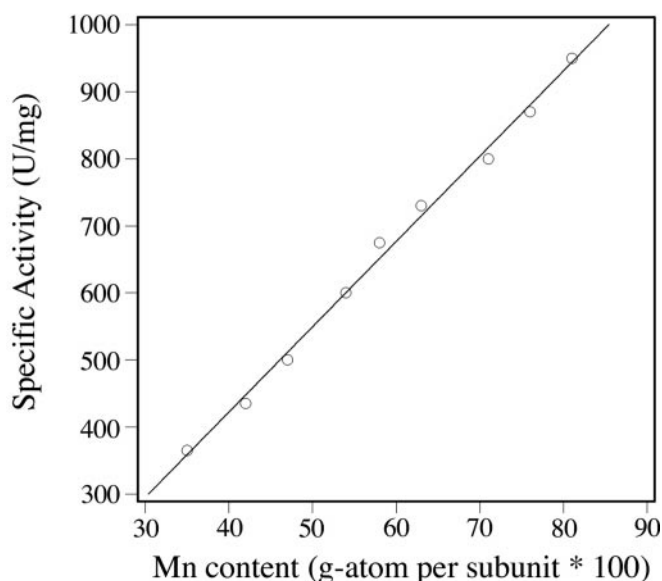


FIG. 1. Correlation between the manganese content and the specific activity of purified, manganese-depleted and reconstituted sweet potato purple acid phosphatase.

the specific activity increased to 800 units/mg (again, the zinc content did not change). We also investigated the possibility of varying the metal ion composition. For mammalian and red kidney bean purple acid phosphatases, a protocol has been established for the generation of metal-free enzyme (5, 16). Although the same treatment does not completely remove the metal ions from sweet potato purple acid phosphatase, short incubations (10–60 s) with the reductant sodium dithionite facilitated the generation of purple acid phosphatase samples with varying manganese content but stoichiometric iron content (1.02 ± 0.06 g-atom/subunit); the amount of zinc present in the samples was below the detection limit (≤ 0.04 g-atom/subunit). The specific activity of these preparations was found to be proportional to the manganese content; this is illustrated in a plot of manganese content *versus* specific activity for purified, manganese-depleted and reconstituted (see below) enzyme (Fig. 1). Clearly, the specific activity increases proportionally with the increase in manganese content in the range between 0.35 and 0.81 g-atom manganese/subunit (correlation coefficient = 0.9952). The calculated specific activity for the enzyme with a full complement of iron and manganese is 1260 units/mg, corresponding to a turnover number for *p*-nitrophenyl phosphate of 1154 s^{-1} .

Second, dialysis of the manganese-depleted enzyme (iron remains stoichiometric) in the presence of MnCl_2 was found to reconstitute enzymatic activity to a value proportional to its manganese content. In contrast, dialysis of the manganese-depleted enzyme against ZnCl_2 , $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$, FeCl_3 , MnCl_3 , or CuSO_4 failed to reactivate the enzyme.

Third, we investigated the possibility that zinc, present as binuclear Fe-Zn centers in sweet potato enzyme preparations, may be responsible for the catalytic activity. The content of zinc ions varies from one preparation to another and is likely influenced by the condition of the soil. Several preparations, which vary in zinc content from below detection limits (≤ 0.04 g-atom) to 0.18 g-atom/subunit (with stoichiometric iron content and ~ 0.6 g-atom of manganese/subunit) exhibit no variation in specific activity (8). Dithionite reduction of purified enzyme (1 g-atom of iron, 0.58 g-atom of manganese, 0.15 g-atom of zinc, and a specific activity of 675 units/mg) and subsequent dialysis against MnCl_2 produced an increase in specific activity (765 units/mg), which correlated with the manganese content (0.68

¹ Slightly better fits were obtained using these g , D , and E values and an $S = 2$ Hamiltonian (e.g. Fe(II) or Mn(III)), but this does not agree with the EPR data.

g-atom/subunit) but not with the zinc (≤ 0.04 g-atom) content. The iron content after dialysis was found to be 0.96 g-atom/subunit. Clearly, the small proportion of binuclear Fe-Zn centers present in the preparations of sweet potato purple acid phosphatase are not responsible for the observed catalytic activity.

Finally, to determine whether Fe(III)-Fe(III) centers are present in sweet potato purple acid phosphatase, we subjected the enzyme to reduction with β -mercaptoethanol under conditions that restored $\sim 90\%$ of the activity of the oxidized pig enzyme. This treatment increased the specific activity of the sweet potato enzyme by only 3%. Similarly, treatment of this enzyme with hydrogen peroxide under conditions that rapidly oxidized and inactivated the Fe(III)-Fe(II) pig enzyme (16) had no effect on the specific activity of the sweet potato enzyme. In the EPR spectrum of the reduced sweet potato purple acid phosphatase (see Supplemental Material, Fig. S1) only very weak resonances attributable to a binuclear Fe(III)-Fe(II) center ($< 2\%$) were observed indicating that at best only a small proportion of sweet potato purple acid phosphatase contains Fe(III)-Fe(III) and (in view of the metal analysis) Mn-Mn centers.

In summary, the above observations clearly indicate that the catalytically active form of sweet potato purple acid phosphatase contains binuclear Fe-Mn clusters. Iron in sweet potato purple acid phosphatase is expected to be most likely in the Fe(III) state because of the similarity of the visible absorption spectra (and the high degree of sequence homology) of the sweet potato and red kidney bean (4) enzymes, which suggests that the band observed at 560 nm in the sweet potato enzyme arises from a tyrosine \rightarrow Fe(III) charge transfer transition. The properties of this novel Fe-Mn center were further investigated using magnetic susceptibility and EPR spectroscopy.

Multifield saturation magnetization of the type used by Day and co-workers (10, 11) on the pig allantoinic fluid purple acid phosphatase was employed to measure the magnetic susceptibility of the enzyme at four magnetic fields (0.2, 1.375, 2.75, and 5 tesla) over a temperature range of 2 to 200 K (Fig. 2). The magnetization values over this temperature range are very weak and show typical Curie behavior (Fig. 2), characteristic of an isolated spin system. The isofield best-fit plots of the experimental susceptibilities (Fig. 2A) employing an $S = 5/2$ spin Hamiltonian produced reasonably good fits and yielded a concentration of 0.082 ± 0.005 mM $S = 5/2$ centers. This concentration corresponds to only $\sim 12\%$ of the total concentration of Fe(III) and Mn(II) centers present in the sample.² For a weakly antiferromagnetically coupled Fe(III)-Fe(II) center (e.g. pig purple acid phosphatase) with $2J = -20$ cm $^{-1}$ ($H = -2JS_1 \cdot S_2$), the susceptibility $\chi = (M/H)$ versus T plot would be expected to show a broad maximum centered at 82 K (Fig. 2B), and this is clearly not observed. Consequently, the weak susceptibility arises from a diamagnetic protein in which the two metal ions (Fe(III), Mn(II)) at the binuclear active site are strongly antiferromagnetically coupled ($-2J \gg 140$ cm $^{-1}$), producing an $S = 0$ ground state.

A low temperature EPR spectrum of sweet potato purple acid

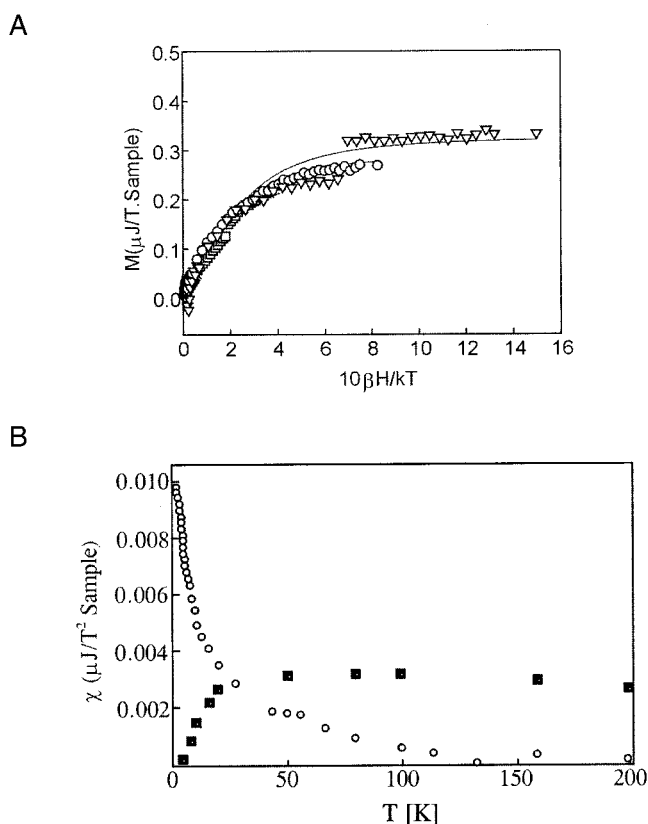


FIG. 2. **Magnetization (M) data for sweet potato purple acid phosphatase.** A, M plotted against $\beta H/kT$ for H values of 0.2 (+), 1.375 (\square), 2.75 (\circ), and 5 T (tesla) (∇) over a temperature range of 2 to 200 K. The solid lines in each isofield set of data were calculated using program WMAG (11) with $g = 2.0$, $E/D = 0.1925$, $D = -1.06$ cm $^{-1}$, and an amount (12.5 ± 0.5 nmol) of $S = 5/2$ centers. The small discontinuity in the 5 T experimental data at $\beta H/kT$ of ~ 7 is probably due to instrumental effects. (Note that the highest value of $\beta H/kT$ measured for each isofield set is the lowest temperature, i.e. $T = 2K \cdot \beta/k = 0.672$ K T^{-1} , where T = tesla. Units for M are microjoules/tesla-Sample.) B, plot of observed susceptibility $\chi (= M/H)$ versus temperature in a field of 2.75 T. Values (\blacksquare) calculated for an $S = 5/2$ dimer with $2J = -20$ cm $^{-1}$ and $g = 2.0$ (23). The values are scaled to the experimental data point at 30 K. Unit conversion: χ (cm 3 ·Sample $^{-1}$) = $10^7 \chi$ ($\mu J/T^2$ ·Sample $^{-1}$).

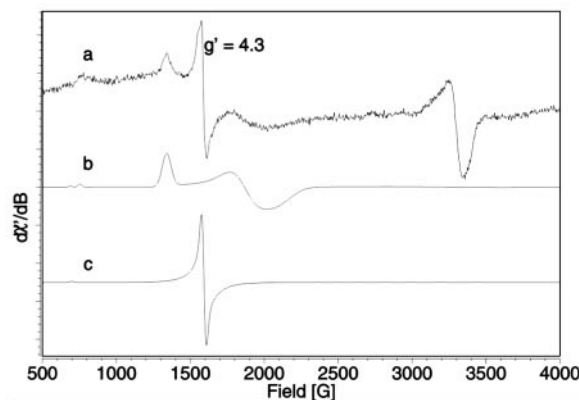


FIG. 3. **EPR spectra of the sweet potato purple acid phosphatase** ($\nu = 9.5784$ GHz, modulation amplitude = 1 mT, $T = 4$ K). a, experimental spectrum revealing resonances characteristic of high spin Fe(III) and Cu(II). $\nu = 9.5784$ GHz, $T = 4$ K. Computer simulation of the component with small (b) and large (c) distributions of E/D . Spin Hamiltonian parameters are given in the text.

phosphatase (Fig. 3) consists of resonances arising from high spin Fe(III) (g' (effective g value) = $3.8 - 9$) and Cu(II) ($g_{\parallel} 2.293$, $g_{\perp} 2.066$, $A_{\parallel} 163.00 \times 10^{-4}$ cm $^{-1}$, $A_{\perp} 8.10 \times 10^{-4}$ cm $^{-1}$). The absence of signals that might reasonably be attributed to

² The excess of iron over manganese, present as 1-iron (Fe(III)) apo-enzyme and/or Fe(III)-Zn(II) centers would then be expected to comprise the $S = 5/2$ signal. This excess (0.20 ± 0.04 mM) is, however, considerably higher than 0.082 ± 0.005 mM. We know from recent work on well characterized $S = 5/2$ Fe(III) rubredoxin samples (17) that the errors between $S = 5/2$ concentration from metal ion analyses and magnetization fits is at most $\pm 15\%$. Fitting attempts that included a combination of Fe(III), $S = 5/2$, and Cu(II), $S = 1/2$ centers yielded reasonable fits but with unreasonably low g values, and the concentrations of Fe(III) were still less than that expected if all of the iron were present in monomeric centers.

either magnetically isolated manganese or a binuclear metal center is consistent with the susceptibility data, indicating a strongly antiferromagnetically coupled center. Computer simulation of the resonances arising from high spin Fe(III) (Fig. 3) were performed using a second-order fine structure spin Hamiltonian ($H = g \beta B \cdot S + S \cdot D \cdot S$) and a distribution of D and E/D values. The distribution of E/D was characterized with five parameters using two Gaussian peaks, although in principle more complicated distributions with more parameters could be used. A good fit was obtained with one Gaussian peak centered at 0.1925 and a half-width of 0.04 and another 50 times smaller at 0.3333 with a half-width of 0.005. The resulting simulations are shown in Fig. 3, *b* and *c*. The presence of these two Gaussian distributions suggests the existence of two components with 1) $E/D = 0.1925$ and 2) $E/D = 1/3$. However, only a very small component with $E/D = 1/3$ is included in the simulation to account for the $g = 4.3$ feature; the spectrum could be reproduced by extending the tail of the broad distribution to $E/D = 1/3$. Therefore, it is more likely that there is a single species present with a large distribution of E/D .

Spin quantitation of the concentration of high spin Fe(III) centers giving rise to the EPR spectrum shown in Fig. 3 is experimentally and theoretically difficult to determine accurately.³ The best way to estimate the spin concentration is to compare the EPR spectra from the red kidney bean and sweet potato enzymes, which suggests that the proportion of sweet potato purple acid phosphatase containing high spin Fe(III) centers (Fe(III)-Zn and Fe(III)-apo) is very small, certainly less than 20%. This is in agreement with the estimate ($\sim 12\%$) from magnetic susceptibility measurements. A comparison of the spin Hamiltonian parameters for the sweet potato enzyme with those for red kidney bean purple acid phosphatase ($D \sim -1 \text{ cm}^{-1}$, $E/D = 0.13$) reveals that the Fe(III) site (i) is more rhombic in the sweet potato enzyme and (ii) arises from either the one-iron apoenzyme or Fe-Zn centers.

In summary, the collective enzymatic and spectroscopic data presented herein indicate the presence of a strongly antiferromagnetically coupled binuclear Fe(III)-Mn(II) center in sweet potato purple acid phosphatase, which is responsible for the enzyme's biological activity.

Model systems containing $\mu\text{-OH-}\mu\text{RCO}_2$ or $\mu\text{-RhO-}\mu\text{-RCO}_2$ bridging combinations in Fe(III)-Mn(II) complexes display weak antiferromagnetism with $2J = 20 \text{ cm}^{-1}$ (19, 21, 22).⁴ As a consequence of the much higher coupling constant, $\mu\text{-oxo}$ Fe(III)-O-Mn(II) bridge(s) are likely to be present rather than a $\mu\text{-hydroxo}$ bridge, which is observed in the Fe(III)-Zn(II) and the Fe(III)-Fe(II) centers of the native and iron-enriched red kidney bean purple acid phosphatase (23). $\mu\text{-Oxo}$ bridges are known in Fe(III) and in Mn(III) compounds but not, to date, in Mn(II) complexes. The seven amino acid residues, which are known to coordinate to the two metal ions in the binuclear centers in pig and red kidney bean purple acid phosphatases, are invariant in the sweet potato enzyme (8). We have recently reported the crystallization of and preliminary x-ray diffraction data on the latter enzyme (24). In a partially refined model of

its crystal structure, it is obvious that these seven invariant amino acid residues bind to the metal ions.⁵ Furthermore, the Fe(III)-Mn(II) internuclear distance at 2.7 Å resolution is significantly shorter (2.9 Å) than in the $\mu\text{-hydroxo}$ bridged enzymes from pig (3.31 Å; Ref. 26) and red kidney bean (3.26 Å; Ref. 27). This observation is consistent with the presence of a $\mu\text{-oxo}$ bridge rather than a $\mu\text{-hydroxo}$ bridge in sweet potato purple acid phosphatase. Studies of additional model complexes with Fe(III)-Mn(II) centers and additional refinement of the crystal structure of the sweet potato enzyme with and without inhibitors are in progress in this laboratory to clarify the interactions in the active site that may contribute to the stabilization of the oxo bridge.

The presence of a $\mu\text{-oxo}$ bridge in the sweet potato enzyme described here may be of great significance for future mechanistic studies on binuclear metallohydrolases. Among all characterized metallohydrolases similarities in metal ion coordination are found, and a common mechanistic theme appears to be present in which the hydrolytic reaction is initiated by an activated solvent nucleophile (28). The identity of the attacking nucleophile remains uncertain. The three-dimensional structure of red kidney bean purple acid phosphatase demonstrates that a terminal Fe(III)-coordinated hydroxide is a likely candidate (27). More recently, metal replacement studies on bovine purple acid phosphatase proposed an alternative mechanism in which a hydroxo ligand of the trivalent metal ion acts as a general base to deprotonate a water molecule in the second coordination sphere, which then attacks the phosphate group of the substrate (29). Analysis of ternary fluoride-enzyme-phosphate complexes in both pig and bovine purple acid phosphatases (30, 31) has led to the suggestion that in the enzyme-substrate complex the phosphate group of the substrate and a hydroxide ion bridge the two metal ions in the active site. It is argued that because the nucleophilicity of this bridging hydroxide is too low for an attack on the phosphate group, the hydroxide is shifted toward the divalent metal ion upon substrate binding. This movement may increase this solvent molecule's nucleophilicity sufficiently for a nucleophilic attack on the phosphorus atom of the substrate (30). However, the oxo bridge in sweet potato purple acid phosphatase is expected to be a significantly stronger nucleophile; hence it cannot be ruled out at present that a direct attack from the bridging oxo moiety initiates the reaction in this enzyme. The differences in metal ion content and bridging ligands in purple acid phosphatases purified from animals (Fe(III)- $\mu\text{-OH-Fe(II)}$), red kidney beans (Fe(III)- $\mu\text{-OH-Zn(II)}$), and sweet potatoes (Fe(III)-X-Zn(II) or Fe(III)- $\mu\text{-O-Mn(II)}$ in different isoforms) may affect the one-electron reduction potentials and substrate specificities, leading to different biological functions *in vivo*.

The discovery of three cDNAs encoding different isoforms of purple acid phosphatase in sweet potato may provide the explanation for some of the variations in metal content and EPR spectra observed by different groups in early work on the enzyme. The enzyme isolated by Hefler and Averill (32) was reported to contain two g-atoms of iron/dimer ($\lambda_{\text{max}} = 545 \text{ nm}$, $\epsilon_{\text{max}} = 3080 \text{ M}^{-1} \text{ cm}^{-1}/\text{Fe}$). The native enzyme showed no evidence for a $g' = 1.70$ signal characteristic of the antiferromagnetically coupled Fe(III)-Fe(II) center of the mammalian purple acid phosphatases, but the authors noted a complex signal in the $g' = 4\text{--}6$ range. The suggestion (32) that the enzyme may contain isolated mononuclear iron centers is now most unlikely in view of the extensive sequence homology between the three sweet potato isoforms and the red kidney bean purple acid phosphatase (7, 8). Durmus *et al.* (7), following a

³ Spin quantitation experiments on high spin Fe(III) centers require an accurate knowledge of the transition probability (normally determined from the simulated spectrum (18)) as this will affect the relative intensities of the individual resonances in the spectrum. Unfortunately, the transition probability cannot be determined uniquely from the computer simulation of the experimental spectrum as the axial zero field-splitting parameter D (-0.8 to -1.2 cm^{-1}) is greater than the microwave quantum. In addition, the distributions of D and E giving rise to the observed line widths cannot be uniquely defined because D and E are unknown.

⁴ C.-J. McKenzie, B. Moubaraki, K. S. and Murray, unpublished data on Fe(III)/Mn(II) model complexes.

⁵ G. Schenk and L. W. Guddat, unpublished results.

purification similar to that of Hefler and Averill (32), showed that their enzyme contains an Fe(III)-Zn(II) center with spectral properties similar to the red kidney bean enzyme. Furthermore, the positions of the resonances attributable to high spin Fe(III) in their enzyme (pH 4.0) are very similar to those seen in the present study (*cf.* Fig. 3a and Durmus *et al.* (7)) indicating, as expected from the sequence homology, that the two forms have similar iron coordination spheres. The sweet potato purple acid phosphatase isolated by Sugiura *et al.* (25) may be different from both the Fe-Mn and the Fe-Zn enzyme. Metal ion analysis of this enzyme indicated the presence of one manganese and negligible iron per 110-kDa dimer. The enzyme exhibited a λ_{max} of 515 nm and $\epsilon_{\text{max}} = 1230 \text{ M}^{-1} \text{ cm}^{-1}$ based on a monomer molecular mass of 55 kDa, values that are significantly different from those of the Fe-Zn and Fe-Mn enzymes. Their enzyme was EPR-silent, but upon denaturation in strong acid (0.8 M HCl, 3 min, 100 °C) resonances centered around $g \sim 2$, typical of Mn(II), were observed. The authors concluded that the native enzyme contained a single Mn(III) coordinated to a tyrosine phenolate. It is feasible that the Mn(II) seen by EPR spectroscopy arose as a result of disproportionation of Mn(III) to Mn(II) and Mn(IV). Although the exact nature of the metal center of the purple acid phosphatase purified by the Japanese group (20, 25) remains unclear, it is possible that it may represent an isoform encoded by the third cDNA, which we and Durmus *et al.* have independently cloned and sequenced but not yet characterized. If so, the metal center is almost certainly a binuclear complex. Possible candidates, despite the reported metal ion analyses, appear to be strongly antiferromagnetically coupled Fe(III)-Mn(II) or Mn(III)-Mn(III), both of which would be EPR-silent and could generate a Mn(II) signal upon denaturation.

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